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The rationale for ovarian cancer prevention is highlighted by the observations that pregnancy and birth control pill use are strongly protective. To achieve a better understanding of the etiology of ovarian cancer, which can then be translated into effective prevention strategies, we have initiated a case-control study that considers genetic susceptibility, epidemiologic risk factors and acquired genetic alterations. Subjects are interviewed in their homes and about 750 cases and 750 controls have been accrued thus far. Blood and cancer samples have been collected and molecular analyses of genetic polymorphisms (BRCA1/2, progesterone receptor, vitamin D receptor, transforming growth factor-beta receptor) have been performed. An initial ovarian cancer chemoprevention trial with levoneorestrel in chickens demonstrated a protective effect and we have shown that progestin mediated apoptosis in the ovarian epithelium is mediated by transforming growth factor-beta. *In vitro* data has suggested that vitamin D analogues may also represent appealing chemopreventives. A chemoprevention trial incorporates both progestins and vitamin D analogues is being initiated. We also are exploring novel therapeutic approaches for targeting the progesterone receptor. These studies have the potential to increase our ability to identify high-risk women and to lead to the development of chemoprevention strategies that might decrease mortality from this disease.

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Introduction

Ovarian cancer is the fourth leading cause of cancer deaths among women in the United States. There are three potential approaches to decreasing ovarian cancer mortality: screening and early detection, more effective treatment and prevention. All of these avenues should be explored, but we believe that prevention represents the most feasible approach. The rationale for prevention is derived from epidemiologic studies that have examined the relationship between reproductive history, hormone use and ovarian cancer. It has been convincingly demonstrated that reproductive events which reduce lifetime ovulatory cycles are protective. Although most women are unaware of this protective effect, those who use oral contraceptive pills for more than 5 years or have 3 children decrease their risk of ovarian cancer by greater than 50%. The biological mechanisms that underlie the association between ovulation and ovarian cancer are poorly understood, however.

Our multidisciplinary ovarian cancer research group has been actively involved in studies that seek to elucidate the etiology of ovarian cancer and to translate this knowledge into effective preventive strategies. Joint consideration of genetic susceptibility, reproductive/hormonal and other exposures, acquired alterations in oncogenes and tumor suppressor genes and protective mechanisms such as apoptosis is required to accomplish this goal. We have initiated a molecular epidemiologic study of ovarian cancer in North Carolina that focuses on the identification of genetic polymorphisms that affect susceptibility to ovarian cancer. Over 1,500 subjects have been accrued thus far in this case-control study. We have examined several polymorphisms and also have forged a collaboration with a group in Australia that is also conducting a DOD funded case-control study of ovarian cancer. This will facilitate progress by allowing us to confirm positive results. In addition, we will pool polymorphism data to increase statistical power to examine relationships with less common histologic types (eg. borderline and non-serous) and gene-gene and gene-environment interactions.

We also are actively involved in development of chemopreventive strategies. We have performed a study in primates that suggests that the oral contraceptive has a potent apoptotic effect on the ovarian epithelium, mediated by the progestin component. In addition, in subsequent studies performed *in vitro*, we have induced apoptosis in epithelial cells treated with the progestin levonorgestrel. Progestin mediated apoptotic effects may be a major mechanism underlying the protection against ovarian cancer afforded by OCP use. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer. Initial studies to test the progestin levonorgestrel in an avian model of ovarian cancer have been undertaken and demonstrated a striking protective effect. In the present study, we are exploring the potential use of vitamin D compounds to enhance the apoptotic effect of progestins on the ovarian epithelium and to enhance the protection against ovarian cancer in the avian model. In addition, we are exploring the molecular pathways (most notably the TGF-beta pathway) that mediate progestin/vitamin D induced apoptosis in the ovarian epithelium. Finally, in an "idea project" we are exploring new pharmacologic approaches to targeting the progesterone receptor for ovarian chemoprevention.

Over the past six years with support from the DOD Ovarian Cancer Research Program we have made considerable progress. This report focuses on the most recent progress in the past 12 months.

Body

Epidemiology and Tissue Core and Project 1: Genetic susceptibility to ovarian cancer

With the support of the Department of Defense Ovarian Cancer Research Program we have initiated a molecular epidemiologic study of ovarian cancer to work towards the goal of a better understanding of the etiology of ovarian cancer. Drs. Andrew Berchuck (Gynecologic Oncologist) and Joellen Schildkraut (Epidemiologist) are working together to lead this study. Our initial plan was to accrue frozen tumor tissue and blood from 500 epithelial ovarian cancer cases treated at Duke University, the University of North Carolina at Chapel Hill and East Carolina University. In addition, 500 age and race-matched control subjects were to be accrued and both cases and controls were to be interviewed by telephone regarding known risk factors for ovarian cancer. After funding to support this project was received from the Department of Defense in 1998 with Dr Berchuck as PI, additional funding was received to support this project in the form of an RO1 grant from the NCI with Dr Schildkraut as PI. The additional funding has allowed us to increase the scope of the study such that nurse interviewers are visiting the homes of all the cases and controls to administer the study questionnaire. Research subjects are now accrued from hospitals in a 48 county region of central and eastern North Carolina using a rapid case ascertainment mechanism established through the state tumor registry. Prior to initiating the study, we had to go through the process of IRB approval in each of the various hospitals involved. The second DOD Ovarian Cancer Program Project which began in 2002 provides funding to increase our accrual to 820 ovarian cancer cases and an equal number of controls. Thus far about 750 women with ovarian cancer and 750 age and race-matched controls have been entered in the study and interviewed. The investigators have project meetings every month with all the research staff to review progress and address ongoing issues and at this point we are pleased with the accrual rate and other procedural aspects of the study. We continue to obtain blood specimens on over 99% of our study subjects. All clinical, epidemiologic and molecular data are stored as they are obtained in a computerized database. Paraffin blocks of tumor tissue are also obtained and these tissues are being used to assess alterations in cancer causing genes such as p53 and HER-2/*neu*. We are continuing to test the hypothesis proposed in the first DOD program project grant that alterations in specific genes may represent molecular signatures that characterize distinct molecular epidemiological pathways of causation of ovarian cancer.

During the study interview a thorough history of the menstrual cycle and reproductive experiences of the study participants is obtained from each subject assisted by the use a life-time calendar method. In addition, information on oral contraceptives and hormone replacement therapy is obtained. Data on the family history of cancer, other risk factors, and potential confounders is also collected. The interview takes 60-90 minutes to complete. The interactions between the nurses and subjects has been uniformly positive. The women with ovarian cancer are highly motivated to talk about their history and have a high level of interest in supporting a study aimed at increasing our understanding of the causes of ovarian cancer. They greatly appreciate the opportunity to talk with a nurse who is truly interested in hearing all the details of their life experience.

Although most of the genes responsible for dominant hereditary ovarian cancer syndromes (BRCA1/2, MSH2/MLH1) likely have been discovered, there is evidence to suggest that polymorphisms in other genes may also affect cancer susceptibility in a more weakly penetrant fashion. In project 1, we are examining the role of genetic susceptibility in the development of ovarian cancer. These studies focus on genes involved in pathways implicated in the development of ovarian cancer. Since the effect of cancer susceptibility genes may be modified by other genes and exposures, he also will determine whether gene-gene and gene-environment interactions affect ovarian cancer susceptibility. Because of the low

incidence of ovarian cancer, the ability to identify "high risk" subsets of women is critical if we hope to translate our emerging understanding of the etiology of ovarian cancer into effective prevention strategies.

BRCA1/2: Since inherited BRCA1or BRCA2 mutations strikingly increase ovarian cancer risk, polymorphisms in these genes could represent low penetrance susceptibility alleles. Prior studies of the BRCA2 N372H polymorphism suggested that HH homozygotes have a modestly increased risk of both breast and ovarian cancer. We have examined whether BRCA2 N372H or common amino acid-changing polymorphisms in BRCA1 predispose to ovarian cancer in the North Carolina ovarian cancer study. Cases included 312 women with ovarian cancer (76% invasive, 24% borderline) and 401 age- and race-matched controls. Blood DNA from subjects was genotyped for BRCA2 N372H and BRCA1 Q356R and P871L. There was no association between BRCA2 N372H and risk of borderline or invasive epithelial ovarian cancer. The overall odds ratio for HH homozygotes was 0.8 (95% CI = 0.4-1.5) and was similar in all subsets including invasive serous cases. In addition, neither the BRCA1 Q356R (OR = 0.9, 95% CI 0.5-1.4) nor P871L (OR = 0.9, 95% CI 0.6-1.9) polymorphisms were associated with ovarian cancer risk. There was a significant racial difference in allele frequencies of the P871L polymorphism (P = 0.64 in Caucasians, L = 0.76 in African Americans, $p<0.0001$). In this population-based, case-control study, common amino acid changing BRCA1 and 2 polymorphisms were not found to affect the risk of developing ovarian cancer. These results were published in Clinical Cancer Research in 2003 (see references).

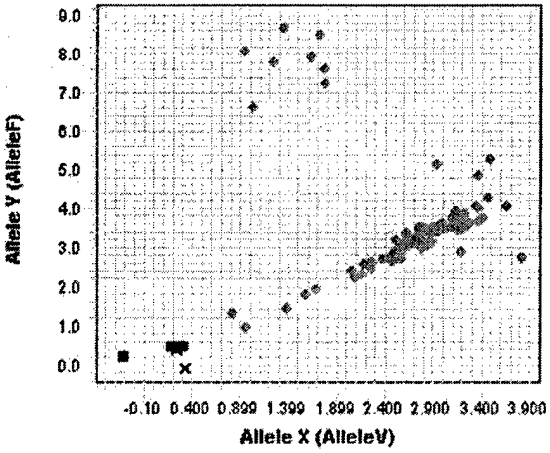
MMP1: It has been suggested that the 2G allele of a guanine insertion/deletion promoter polymorphism in the promoter of the matrix metalloproteinase-1 (*MMP1*) gene may increase susceptibility to ovarian cancer. The 2G allele also has been associated with increased *MMP1* expression. We investigated the relationship between the *MMP1* polymorphism and ovarian cancer risk in a large population-based, case-control study. The *MMP1* promoter polymorphism was examined in white blood cell DNA from 311 cases and 387 age-and race-matched controls using a radiolabeled PCR assay. In addition, genotyping of the *MMP1* polymorphism performed in 42 advanced stage invasive serous ovarian cancers was compared to their mean relative *MMP1* expression from Affymetrix microarrays. The 2G allele frequency did not differ significantly between cases (0.49) and controls (0.48) and the distribution of genotypes was in Hardy-Weinberg equilibrium. Using 1G homozygotes as the reference group, neither 2G homozygotes (OR 1.1, 95% CI 0.7-1.7) nor heterozygotes plus 2G homozygotes (OR 0.9, 95% CI 0.7-1.3) had an increased risk of ovarian cancer. There was also no relationship between *MMP1* genotype and histologic grade, histologic type, stage, or tumor behavior (borderline vs. invasive). The mean *MMP1* expression was twice as high in 2G homozygotes relative to 1G homozygotes, but this difference was not statistically significant. The reported association between the *MMP1* promoter polymorphism and ovarian cancer risk is not supported by our data. There was a suggestion that the 2G allele may be associated with higher *MMP1* expression and this finding is worthy of further investigation. These results were published in 2003 in the Journal of the Society for Gynecologic Investigation (see references).

Progesterone receptor: In view of the protective effect of a progestin dominant hormonal milieu (OC use, pregnancy), progesterone receptor variants with altered biological activity might affect ovarian cancer susceptibility. A German group reported that an intronic insertion polymorphism in the progesterone receptor was associated with a 2.1-fold increased ovarian cancer risk. It subsequently was shown that this *Alu* insertion is in linkage disequilibrium with SNPs in exons 4 and 5. However, several subsequent studies by our group and others failed to confirm an association between these polymorphisms and ovarian cancer. In addition, there is little evidence that this complex of polymorphisms, termed PROGINS, alters progesterone receptor function.

More recently, sequencing of the progesterone receptor gene has revealed several additional polymorphisms, including one in the promoter region (+331G/A). The +331A allele creates a unique

transcriptional start site that favors production of the progesterone receptor B (PR-B) isoform over progesterone receptor A (PR-A). The PR-A and PR-B isoforms are ligand-dependent members of the nuclear receptor family that are structurally identical except for an additional 164 amino acids at the N-terminus of PR-B, but their actions are distinct. The full length PR-B functions as a transcriptional activator and in the tissues where it is expressed it is a mediator of various responses, including the proliferative response to estrogen or the combination of estrogen and progesterone. PR-A is a transcriptionally inactive dominant-negative repressor of steroid hormone transcription activity that is thought to oppose estrogen-induced proliferation. An association has been reported between the +331A allele of the progesterone receptor promoter polymorphism and increased susceptibility to endometrial and breast cancers. It was postulated that upregulation of PR-B in carriers of the +331A allele might enhance formation of these cancers due to an increased proliferative response.

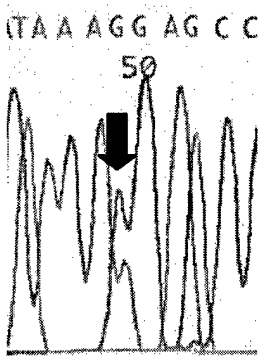
The +331G/A polymorphism in the progesterone receptor promoter was examined in cases and controls from the North Carolina Ovarian Cancer Study. A second, independent, case-control study from Australia (Dr. Chenevix-Trench) that is also funded by the DOD was examined to confirm associations seen in the North Carolina study. Data from the two studies was then pooled to increase statistical power. The +331G/A single nucleotide polymorphism in the promoter of the progesterone receptor was genotyped using a TaqMan assay. Allelic discrimination was performed using the MGB primer/probe TaqMan assay on the ABI Prism 7700 system. Some samples were sequenced using the ABI 3100 system to confirm the accuracy of the Taqman assay. The +331A allele was found in 59/504 (11.7%) Caucasian controls and the distribution of genotypes was in Hardy-Weinberg Equilibrium (χ^2 = 0.391, p = 0.53). Only 1/81 (1.2%) African American controls and none of 67 African American women with ovarian cancer carried the +331A allele. In view of the rarity of the +331A allele in African Americans, these subjects were excluded from further analyses. The +331AA homozygotes were combined with heterozygotes in calculating odds ratios. The +331A allele was associated with a modest reduction in risk of ovarian cancer. Analysis by histologic type revealed that there was a slight trend towards protection against the common serous histologic type (OR = 0.80, 95% CI 0.49–1.29) but there was a more striking protection against endometrioid and clear cell cancers (OR = 0.30, 95% CI 0.09–0.97).



PR promoter polymorphism

(left) TaqMan assay (green = GA heterozygotes, red = GG homozygotes)

(right) GA heterozygote



Relationship between PR promoter polymorphism and ovarian cancer risk in histologic types of ovarian cancer

	PR +331 G/A Genotype							
	GG	AG	AA	AG/AA		OR	(95% CI)	
Controls	445	58	1	59	(11.7%)	1.00	Reference	
Serous	244	26	0	26	(9.6%)	0.81	(0.50 -	1.32)
Mucinous	44	5	0	5	(10.2%)	0.80	(0.30 -	2.14)
Endometrioid	53	3	0	3	(5.4%)	0.43	(0.13 -	1.40)
Clear cell	23	0	0	0	(0.0%)	**		
Endometriod/ clear cell	**76**	**3**	**0**	**3**	**(3.8%)**	**0.30**	**(0.09 -**	**0.97)**

In view of the potential for false-positive results in genetic association studies, confirmation was sought using an independent study population from Australia. The frequency of the +331A allele among Caucasian controls varied by less than 1% between the Australian and North Carolina studies. The Australian study was not a population-based case-control study and fewer data were available regarding risk factors. Nevertheless, the results of the Australian study were similar to those of the North Carolina study, with a modest overall protective effect that was most pronounced for endometrioid cancers (OR = 0.51, 95% CI = 0.17–1.53). The Breslow-Day chi-square test was used to assess homogeneity of the results from the two study populations. Analyses involving the combined data set showed a significant association between the +331A allele and decreased risk of endometrioid/clear cell cases. In combining the two studies there was a significant risk reduction (OR = 0.46, 95% CI = 0.23–0.92) (P = 0.027). These types represent 21% of invasive ovarian cancer cases. Endometriosis is known to increase risk of endometrioid and clear cell ovarian cancers, many of which may arise in ovarian deposits of endometriosis. In this study, endometriosis was associated with an increased risk of endometrioid/clear cell cancers (OR = 3.87, 95% CI = 2.09-7.17. The +331A allele appeared to be strongly protective against endometriosis (OR = 0.19, 95% CI 0.03 – 1.38), but this study was under powered to prove this conclusively.

The finding that the +331A allele was associated with a decreased risk of endometrioid and clear cell ovarian cancers was somewhat unexpected in view of prior reports of an increased risk of endometrial and breast cancers in carriers of the +331A allele. We also observed preliminary evidence that this polymorphism may protect against endometriosis, the precursor of many of these cancers. Endometriotic implants have been shown to express only the PR-A isoform, and it has been suggested that the absence of PR-B may account for the lack of appropriate cycling of these glands. In normal cycling endometrium PR-A expression is predominant during the proliferative phase whereas a shift towards PR-B occurs with differentiation in the early secretory phase. Since the +331A allele of the PR promoter polymorphism favors production of the PR-B isoform, it is possible that this might prevent the PR-A:PR-B imbalance in endometriotic implants and protect against the growth and spread of endometriosis to the extent that it becomes clinically apparent. The reduced risk of endometrioid and clear cell cancers in women with the +331A allele might be attributable to a lower likelihood of carriers developing more extensive endometriosis that serves as a precursor for these cancers. In contrast to the pathogenic model proposed for endometriosis in which the +331A allele counters an abnormal imbalance in the PR-A:PR-B ratio, in normal breast and endometrial tissues the polymorphism may create an imbalance that enhances both the proliferative response to estrogen and cancer risk.

The literature is fraught with false-positive association studies of genetic susceptibility polymorphisms, but several features mitigate the likelihood of this in the present study. First, the known protective benefit of progestins against ovarian cancer provides a preexisting biologic plausibility for the observed association. In addition, the finding that the +331A allele is protective against both endometrioid/clear cell cancers and their precursor lesion (endometriosis) also is supportive. Confirmation of the positive association obtained in North Carolina study by the Australian study also represents an additional critical validation step. Finally, unlike many polymorphisms that lack known functional significance, the +331A allele increases transcription of PR-B *in vitro*. This study provides evidence for the existence of low penetrance ovarian cancer susceptibility polymorphisms. If multiple polymorphisms are identified that either increase or decrease the risk of various histologic types of ovarian cancer, this might be used in the future for risk stratification that would facilitate screening and prevention strategies.

The paper describing the relationship between the progesterone receptor promoter polymorphism and ovarian cancer will be published in the December 2004 issue of Cancer, Epidemiology, Biomarkers and Prevention (see appendix).

TGF-β receptor 1: Progestin induced apoptosis in the ovarian epithelium may be mediated by the TGF-β pathway, and this pathway is the target for chemopreventive efforts in Project 2. In project 1, we are investigating the possibility that TGF-β receptors are appealing candidate ovarian cancer susceptibility genes. A polymorphism in the TGF-β I receptor has been described that involves deletion of 3 alanines from a 9 alanine tract (TβR1(6A)). IT has been suggested that the 6A allele might predispose to the development of ovarian cancer and other cancer types. In addition, there is some evidence that the TβR1(6A) variant may be functionally significant and may confer an impaired ability to mediate TGF-β anti-proliferative effects.

In view of the evidence that the TGFβR1 polyalanine polymorphism may affect ovarian cancer risk, this polymorphism was genotyped in 588 ovarian cancer cases and 614 controls from the North Carolina study (see tables below). Significant racial differences in the frequency of the 6A allele were observed between Caucasian (10.7%) and African American (2.4%) controls ($p<0.001$). One or two copies of the 6A allele of the TGFβR1 polyalanine polymorphism were carried by 18% of all controls and 19% of cases, and there was no association with ovarian cancer risk (OR = 1.07, 95% CI 0.80 – 1.44). The odds ratio for 6A homozygotes was 1.81 (95% CI 0.65 – 5.06), but these comprised only 0.98% of controls and 1.70% of cases. The 6A allele of the TGFβR1 polyalanine polymorphism does not appear to increase ovarian cancer risk. Larger studies are needed to exclude the possibility that the small fraction of individuals who are 6A homozygotes have an increased risk of ovarian or other cancers. Polymorphisms in other members of the TGF-β family of ligands, receptors and downstream effectors also are appealing candidates. This data was communicated as an oral presentation at the 2004 meeting of the International Gynecologic Cancer Society in Scotland. A manuscript has been submitted for publication.

Demographic and clinical features of ovarian cancer cases and controls in the North Carolina Ovarian Cancer Study

	Cases (N=588)		Controls (N=614)		
Age in years					
mean (s.d)	54.1	(11.5)	54.8	(12.3)	
median (range)	54	(20-74)	54	(20-75)	
	n	**(%)**	**n**	**(%)**	**P value**
Race					
Caucasian	495	(84)	520	(85)	
African-American	77	(13)	83	(14)	
Other	16	(3)	11	(2)	
Menopause status					
Pre/Peri	226	(39)	248	(40)	0.55
Post	361	(61)	366	(60)	
Tubal ligation					
No	443	(75)	403	(66)	<0.001
Yes	144	(25)	211	(34)	
Oral contraceptive use (months)					
None	208	(35)	196	(32)	0.09
≤ 12	101	(17)	100	(16)	
> 12	265	(45)	309	(50)	
user of unknown duration	13	(2)	9	(1)	
Livebirths					
0	123	(21)	81	(13)	<0.001
1	105	(18)	94	(15)	
>1	359	(61)	439	(71)	
Family History of Ovarian Cancer					
No	562	(96)	596	(97)	0.04
Yes	25	(4)	17	(3)	
Tumor Behavior					
Borderline	133	(23)			
Invasive	454	(77)			

Histologic Subtype		
Serous	353	(60)
Endometrioid	71	(12)
Mucinous	70	(12)
Clear Cell	37	(6)
Other	57	(10)
Stage		
I	208	(35)
II	42	(7)
III	310	(53)
IV	19	(3)
Unknown	9	(2)

* Odds ratios are age and race adjusted.

1 missing tumor behavior and 5 missing stage

Relationship between *TGFBRI* polymorphism and ovarian cancer risk in Caucasian and African-American subjects

	All Races					
	Controls		Cases			
Genotype	**n**	**(%)**	**n**	**(%)**	**OR ***	**95% CI**
9A/9A	497	(81%)	468	(80%)	1.00	reference
6A/6A	6	(1%)	10	(2%)	1.81	(0.65- 5.06)
6A/9A	104	(17%)	100	(17%)	1.03	(0.76- 1.40)
6A/6A or 6A/9A	110	(18%)	110	(19%)	1.07	(0.80- 1.44)
Other	7	(1%)	10	(2%)	1.71	(0.62- 4.70)

*age and race adjusted

**age adjusted

Vitamin D Receptor pathway: High circulating levels of vitamin D may protect against ovarian cancer, since mortality rates are higher in northern latitudes where there is less sunlight. The most biologically active form of vitamin D, 1,25 $(OH)_2D_3$, is produced in the skin through sunlight exposure and vitamin D exhibits significant antineoplastic properties. Several factors, both dietary and genetic regulate the production of 1,25 $(OH)_2D_3$ from its precursor. A recent study suggested that about 22% of the variation may be accounted for by a putative major gene effect. Highly polymorphic loci involved in the metabolism and function of vitamin D include the vitamin D binding protein and vitamin D receptor genes. It has been suggested that a polymorphism in the vitamin D receptor gene involving a shared haplotype that includes a change in the 3' untranslated region that alters transcriptional activity may be associated with increased prostate cancer risk. This has not been a uniform finding in all studies, however.

Vitamin D receptor polymorphisms are being examined in the North Carolina Ovarian Cancer Study to test the hypothesis that vitamin D biosynthesis in the skin can protect susceptible individuals from

developing ovarian cancer and that genetic variation in the vitamin D pathway may modify this protective effect. Preliminary data has been obtained in the past week using three tagging single nucleotide polymorphisms that define the major haplotypes of the vitamin D receptor gene (see below). This data is presently being subjected to more detailed analysis of the relationship between various haplotypes and risk of various subsets of ovarian cancer in blacks and whites. Polymorphisms in other genes in this pathway such as the vitamin D binding protein also will be examined.

In view of the potential protective effect of sunlight, the efficacy of vitamin D analogues is being examined in the chemoprevention studies outlined in project 2.

HAPLOTYPE ANALYSIS OF VITAMIN D RECEPTOR GENE POLYMORPHISMS AND OVARIAN CANCER RISK IN CAUCASIAN AND BLACK CASES AND CONTROLS

	WHITES				BLACKS			
	cases	**%**	**controls**	**%**	**cases**	**%**	**controls**	**%**
VDRAPA1								
AA	148	(29)	161	(29)	22	(28)	37	(42)
AC	268	(52)	272	(48)	41	(52)	46	(52)
CC	95	(19)	129	(23)	16	(20)	6	(7)
VDRFOK1								
AA	61	(12)	75	(13)	5	(6)	3	(3)
AG	249	(48)	269	(48)	24	(31)	27	(30)
GG	206	(40)	218	(39)	49	(63)	59	(66)
VDRTAQ1								
CC	82	(16)	98	(17)	6	(8)	6	(7)
CT	272	(53)	254	(45)	26	(33)	34	(38)
TT	163	(32)	209	(37)	47	(59)	49	(55)

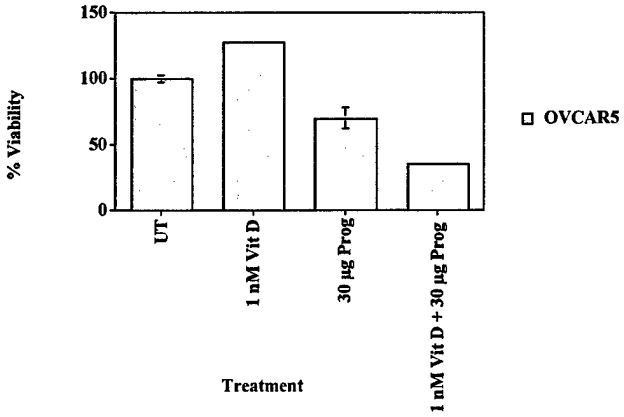
Project 2: Chemoprevention of Ovarian Cancer

Project 2 is under the direction of Gustavo Rodriguez, M.D. (Gynecologic Oncologist). The prevention strategy outlined in our proposal focuses on the potential use of a combined approach incorporating both progestins and Vitamin D for the chemoprevention of ovarian cancer. The studies outlined in our prevention grant are designed to add further support to notion that progestins and Vitamin D are potent apoptotic agents on human ovarian epithelial cells, and to directly test the hypothesis in an animal model these agents confer preventive effects against ovarian cancer. These aims in the grant are: (1) to evaluate the apoptotic effect of progestins and vitamin D analogues on the human ovarian epithelium *in vivo*, (2) elucidate the molecular mechanisms by which they induce apoptosis in ovarian epithelial cells, and (3) to directly test the hypothesis that progestins/vitamin D analogues confer preventive effects against ovarian cancer in a chemoprevention trial in the chicken, the only animal species with a high incidence of ovarian cancer.

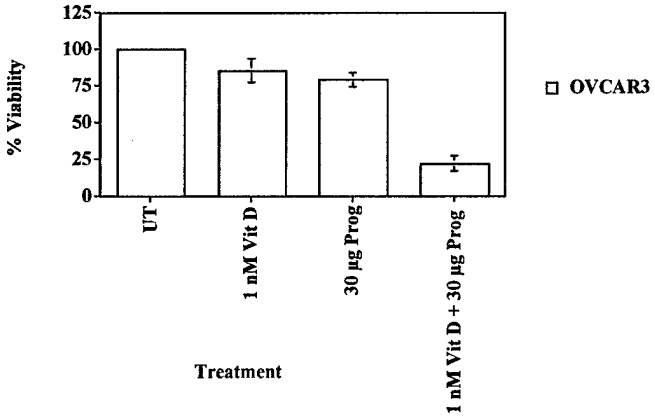
There is significant potential to decrease ovarian cancer incidence and mortality through prevention. Epidemiological evidence has shown that routine use of the combination estrogen–progestin oral contraceptive pill (OCP) confers a 30-50% reduction in the risk of developing subsequent epithelial ovarian cancer, suggesting that an effective ovarian cancer preventive approach using hormones is possible. Investigations by our group have elucidated a mechanism that we believe is responsible for the ovarian cancer preventive effects the OCP. Specifically, we have discovered that the progestin component of the OCP is functioning as a classic chemopreventive agent, by activating potent molecular pathways known to be associated with cancer prevention in the ovarian surface epithelium. We have discovered that progestins markedly induce programmed cell death (apoptosis) and differentially regulate expression of Transforming Growth Factor Beta (TGF-β) in the ovarian epithelium. These two molecular events have been strongly implicated in cancer prevention *in vivo*, and are believed to underlie the protective effects of other well-known chemopreventive agents such as the retinoids and Tamoxifen. Our laboratory and animal research findings are supported by human data demonstrating that progestin-potent OCPs confer twice the ovarian cancer protection as newer weak-progestin OCPs. These human data provide proof of principle that progestins are effective chemopreventive agents for ovarian cancer, and suggest that a regimen that has enhanced chemopreventive biologic potency in the ovarian epithelium will be more effective than a lower potency regimen for ovarian cancer prevention.

The finding that progestins activate these molecular pathways in the ovarian epithelium opens the door toward a further investigation of progestins as chemopreventive agents for ovarian cancer, and raises the possibility that other agents that similarly activate cancer preventive pathways in ovarian epithelial cells may be attractive ovarian cancer preventives. Among the non-progestins, there is environmental, epidemiologic, laboratory and animal evidence in support of Vitamin D as a potent ovarian cancer preventive. In addition, results from a prevention trial that we have performed in the chicken ovarian cancer animal model suggest an additive ovarian cancer protective effect of Vitamin D when added to progestin. In last year's report, we presented evidence of *in vitro* experiments showing that the combination of a progestin and Vitamin D had a more potent biologic effect on cells derived from the human ovarian epithelium than either agent alone. The figures below demonstrate a marked impact on cell viability when the two agents are combined, and administered at a dosage that has a marginal impact for each agent given alone. Subsequent studies that we have performed over the past year have demonstrated that the effect is synergistic.

Effect of Vitamin D and Progesterone on OVCAR5 Cells



Effect of Vitamin D and Progesterone on OVCAR3 Cells



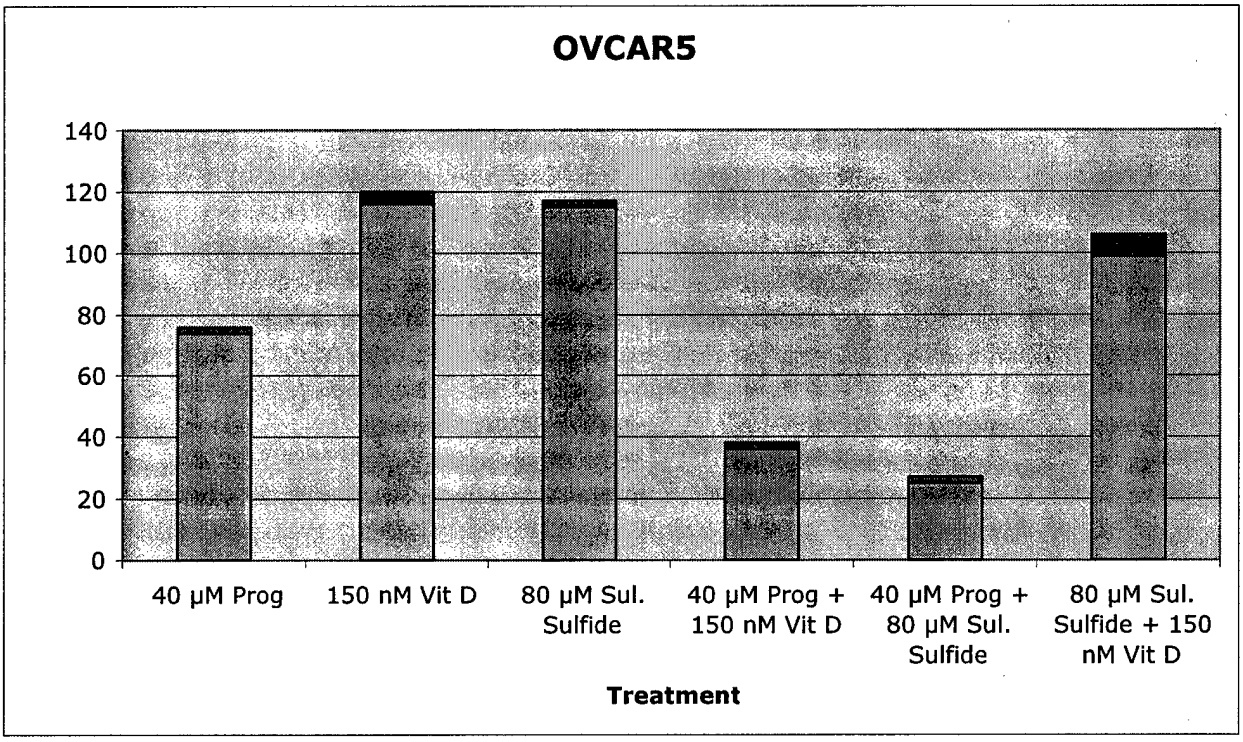
Based on these findings, we hypothesize that progestins and Vitamin D target the early steps of carcinogenesis in the ovarian epithelium, by activating apoptosis and thereby decreasing dysplastic ovarian epithelial cells, resulting in effective cancer prevention. In addition, we hypothesize that the combination of two preventive agents such as progestin plus Vitamin D will be a more potent ovarian cancer preventive than either agent used alone, making it possible to lessen the dose of each in order to achieve optimal chemoprevention, while minimizing side effects.

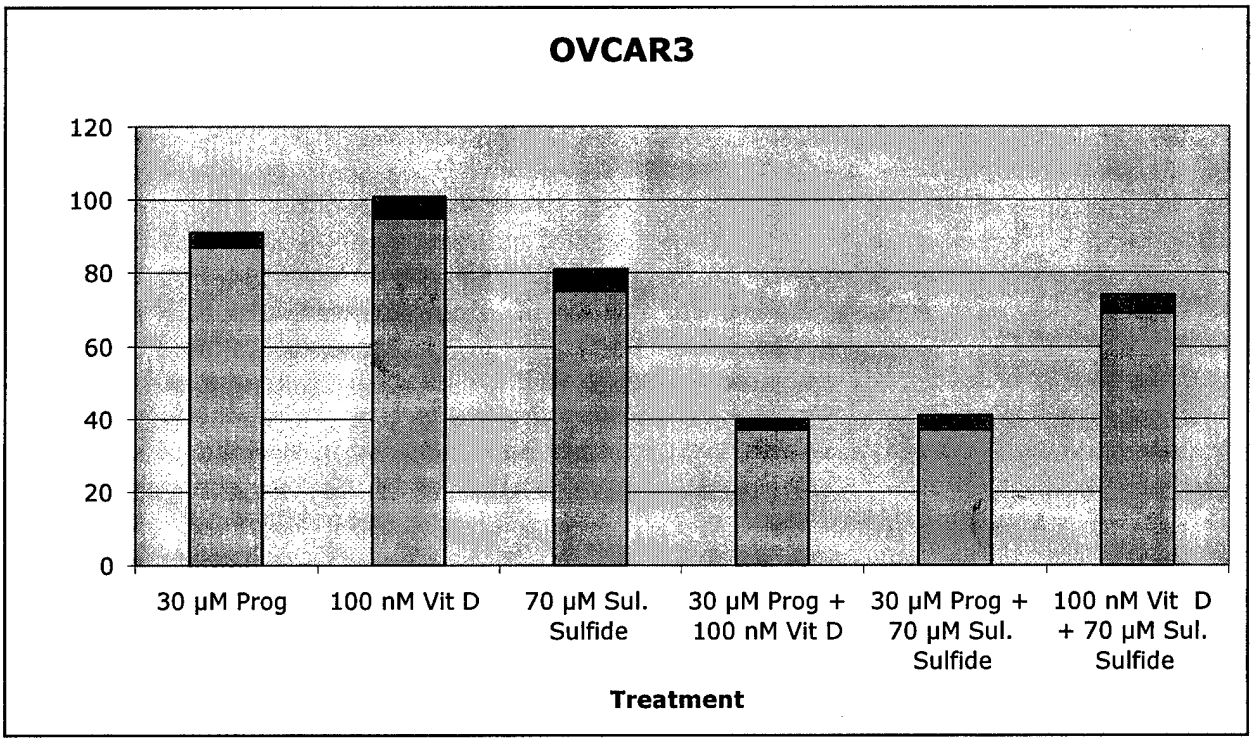
Molecular Mechanisms Underlying the Biologic Effects of Progestins and Vitamin D on the ovarian epithelium: There is a growing body of literature unraveling the biologic influence of hormones on epithelial cells. Hormones have long been thought to exclusively work by binding to specific receptors, which then undergo conformational changes and translocate to the nucleus regulate transcription. Experiments are underway to examine the genomic effects of Vitamin D and progestins, or the combination on the ovarian epithelium. RNA has been collected from ovarian cell lines treated with progestins and Vitamin D and microarrays are being performed to use a high throughput approach to examine the impacts of these hormones on apoptotic and TGF-beta signaling pathways. Importantly, we hope to identify the signaling events underlying the synergy that we observe when the two hormones are combined. We hope to use the microarray experimental results to direct our efforts in pursuing the signaling pathways of Vitamin D and progesterone toward apoptosis. For instance, a recent publication demonstrates that Vitamin D lowers telomerase expression in OVCAR3 cells preceding the activation of the caspase cascade (Jiang F. et al. 2004, J Biol Chem epub Oct 12, 2004). Telomerase is a very exciting molecule to study, as it is always expressed in stem cells, not expressed at all in healthy somatic cells, and frequently expressed in tumor cells. Also, using microarrays to study breast cancer cell lines treated with Vitamin D, Swami et al. (2003 Breast Canc Res Treat 80:49-62) have demonstrated that Vitamin D up regulates TGF-beta 2, an isoform we have identified as being up regulated by progesterone en route to apoptosis in normal primate ovarian epithelium (J Natl Cancer Inst 2002 9(1):50-60).

In addition to the classic genomic view of hormonal action in cells, hormones are now known to cause non genomic effects as well. Non genomic effects are defined as those effects that are rapid (within minutes), are not affected by the addition of actinomycin D or cycloheximide, and can occur in cells lacking the classic receptor of the hormone being studied (for review see Losel R and M Wehling, 2003 Nat Rev Mol Cell Biol 4(1):46-56). For instance, Vitamin D, progesterone, estrogen and other hormones are able to rapidly activate ion channels, kinases and second messengers that affect the operations of the cell without requiring transcription. In addition, progesterone can intercalate into the lipid bilayer of ovarian adenocarcinoma cells (McDonnel AC et al. 2003, Exp Biol Med 228:308-14). Once there it decreases membrane fluidity, affecting exocytosis, cellular invasiveness, ion flux and signaling, all independently of binding to its receptor.

To help us understand whether we need to concentrate on genomic or nongenomic responses to Vitamin D and progestin, we made cell lysates of our cell lines and looked for expression of the Vitamin D receptor or progesterone receptors by Western blot. In all cases we were able to identify the Vitamin D receptor, indicating that at least some of the Vitamin D response will be genomic. Using the culture conditions in which we grow and treat the cells, we have seen variable expression of the progesterone receptor, perhaps hinting at nongenomic mechanisms of action with this hormone.

In addition to studying the chemopreventive effects of Vitamin D and progesterone, also have been studying the role NSAIDs might play in preventing ovarian cancer. We have been able to demonstrate that the NSAIDs Celecoxib and Sulindac sulfide cause cellular apoptosis in ovarian cancer cell lines (OVCAR3 and OVCAR5), in addition to immortalized human ovarian surface epithelial cells (H10-188V) and a primary culture of normal human ovarian epithelial cells (NOE; E6-transformed NOE cells are named NOE 712). When very low toxic doses of progesterone and NSAID are added to the cells, we see a synergistic activation of apoptosis, similar to the response we see when we add very low toxic doses of Vitamin D and progesterone to the cells. However, very low toxic doses of Vitamin D and NSAID in combination do not cause any enhancement in cell death (see figures below).


OVCAR5
140
120
100
80
60
40
20
0
40 µM Prog
150 nM Vit D
80 µM Sul. Sulfide
40 µM Prog + 150 nM Vit D
40 µM Prog + 80 µM Sul. Sulfide
80 µM Sul. Sulfide + 150 nM Vit D
Treatment


OVCAR3
120
100
80
60
40
20
0
30 µM Prog
100 nM Vit D
70 µM Sul. Sulfide
30 µM Prog + 100 nM Vit D
30 µM Prog + 70 µM Sul. Sulfide
100 nM Vit D + 70 µM Sul. Sulfide
Treatment

One possible explanation for this result is that both the Vitamin D and the Sulindac sulfide may be pro-apoptotic through the same mechanism, and there is no benefit to be gained through their combination at these doses. For instance, as mentioned earlier, Vitamin D has now been shown to down regulate telomerase expression in OVCAR3 cells. There are also reports that non-steroidal anti-inflammatory drugs (NSAIDs) can down-regulate telomerase expression in an apparently non-cox-mediated fashion (Baoping Y. et al. 2004 Dig Dis Sci 49(6):948-53). Telomerase is one molecule we will begin to examine in our various cell lines, to see if it is directly affected by addition of Vitamin D, progesterone or an NSAID. Using the real-time PCR techniques we have worked out to measure changes in TGF-beta transcription levels, we can look for basal expression of telomerase and then any changes in its expression following treatments.

We plan to continue unraveling how Vitamin D causes apoptosis in our ovarian cell lines, keeping in mind that it may be exerting both genomic and nongenomic changes. We will use microarray to examine the genomic changes, using untreated cells, Vitamin D treated cells, and cells exposed to the combinations of Vitamin D and progesterone, and Vitamin D and Sulindac sulfide. Determination of transcriptional changes following treatments will be very useful in defining signaling pathways to pursue. We can compare the transcriptional changes in treated cell lines that are already cancerous (OVCAR3 and OVCAR5) to normal transformed cells (H10-118V and NOE 712) to see if different sets of genes are activated or repressed, depending on the type of cell. We will also exploit our cell viability data to try to uncover why Vitamin D and progesterone are synergistic, and why Vitamin D and NSAIDs are not. Understanding what happens when various classes of chemopreventive agents are combined will be very useful for designing future clinical trials, aimed at optimal prevention of a disease like ovarian cancer.

Evaluation of Progestin and Vitamin D for Ovarian Cancer Chemoprevention in the Chicken

The planned chemoprevention trial is scheduled to begin 11/29/04. A baseline necropsy has been performed on 800 two-year old birds from the study flock. Over 3600 birds will be randomized into 6 groups, including

1) Control (contains baseline recommended allowance of Vitamin D)
2) High dose Vitamin D (5x the amount of D in group one)
3) High dose progestin
4) Low dose progestin
5) High dose progestin plus High dose D
6) Low dose progestin plus High dose D

The Vitamin D to be used will be 25-OH D3. The baseline vitamin D requirement is satisfied at 0.03125 mg/lb of feed. This is reflected in the diets that are formulated for groups 1,3,and 4. Groups 2,5,and 6 will receive a 5x dose, or 0.156 mg/lb of feed. The low progestin dose group will receive .05 mg/day Levonorgestrel equivalent (same as first chicken trial demonstrating a chemopreventive effect), and the high progestin dose group will receive .5mg/day Lev the dose Levonorgestrel equivalent.

The is designed with sufficient sample size for adequate power to detect the subtle differences between the treatments and accounting for expected mortality during the trial, based on our experience with a similar flock and conditions in our first trial. The experimental design is a factorial and is properly balanced and easily analyzed. We hope to demonstrate dose response effects and this is the rationale for the low and high dose D and progestin design. Also the design will allow us to look for synergistic effects, especially with the low D and low P groups.

In the chicken, 1α25-OH-D_3 is 6x more active than D_3. In the chicken Vitamin D3 is converted in the liver to 25OH D3 then in the Kidney to either 24,25-(OH)2-D3 or to 1α25-OH-D_3 depending on the parathyroid hormone level. The 24,25(OH)2-D3 goes through two additional rearrangements to get to 1α25-OH-D_3 that functions as a hormone. The vitamin D3 has a half life in the bird of approximately 25 days where as the half life of the more active forms can be as short as 6 hr.

Idea Project: Probing the mechanism(s) of crosstalk between estrogen and progesterone signaling pathways: A first step in the search for novel chemopreventatives

Donald P. McDonnell, Ph.D.

Ovarian cancer is the fourth leading cause of cancer deaths in Western countries and the most fatal gynecological cancer (1). The ovary is the main site of sex-steroid hormone production in females, and recent studies indicate a role for estrogen in ovarian cancer. Ovarian surface epithelial cells, the site of 90% of malignancies, show a marked proliferative response to estrogens (2). Analysis of primary tumor samples has revealed that as many as 70% of ovarian cancers express estrogen receptors (ERs), which confers estrogen responsiveness (3, 4). The ER-α is thought to mediate the mitogenic actions of estrogen by inducing the expression of genes involved in cell proliferation. However, in contrast to other ER+ tissues (breast, uterus), there is a notable lack of regulation of classical estrogen-responsive genes (PR, c-fos, pS2) in ovary and ovarian cancer cells (5).

The ability to link proliferation to specific gene changes has been difficult, as several groups have demonstrated that there are hundreds of primary and secondary responses to estrogens in ovarian and breast cancer cells treated with estradiol. The inability to satisfactorily annotate the gene expression patterns identified has necessitated a candidate gene approach in defining the key genes required for proliferation. It was in this manner that we recently identified stromal cell derived factor-1 (SDF-1), a growth-stimulatory chemokine, as a key target of estrogens in ER-positive ovarian and breast cancer cells (6). Specifically, SDF-1 was shown to be a primary target of ER and upon estradiol treatment, both the SDF-1 mRNA and secretion of its corresponding chemokine was increased. Neutralizing antibodies to SDF-1 blocked the mitogenic actions of estradiol whereas activation of the SDF-1 receptor CXCR4 obviated the need for estradiol supplementation. Importantly, these data define at least one genomic response that is required for estrogen-stimulated cell proliferation.

The discovery that induction of the SDF-1-CXCR4 regulatory axis by ER is a key event in ovarian cancer cell growth suggested agents that inhibit SDF-1 expression might be effective in suppressing estrogen-induced proliferation of ovarian cancer cells. BG-1 epithelial ovarian and MCF-7 breast cancer cells were used to screen for such inhibitors, as both cell lines express functional ER and progesterone receptor (PR), grow in response to estrogens, and are the sources in which the original link between estrogen and the SDF-1 pathway was defined (6). By treating BG-1 cells or MCF-7 breast cancer cells with a series of pharmacological agents and monitoring endogenous SDF-1 expression, several small molecule inhibitors of this regulatory pathway were identified. As expected, antiestrogens were capable of suppressing SDF-1 induction by estrogen as shown previously (6). However, since antiestrogens are not useful in blocking ER action in a physiological settings due to the high level of estrogens in the ovary, we chose to focus on two other classes of SDF-1 inhibitors identified in our screen, (I) progestins and (II) ligands for peroxisome proliferator-activated receptor γ (PPARγ).

In the discovery of Class I agents, we showed that progestin-activated PRs effectively inhibited SDF-1 induction by estrogens in both BG-1 and MCF-7 cells (7). In probing the mechanism, it was found that

PR inhibits ER induction of SDF-1 and a series of other known estrogen-inducible genes by directly interfering with ER activation on target gene promoters. We have therefore hypothesized that progestins may be growth-inhibitory in some circumstances by opposing estrogen in regulation of SDF-1 expression and perhaps other ER targets. The impact of this functional interaction between ER and PR signaling on ovarian cancer cell proliferation is an ongoing area of investigation.

Class II inhibitors of SDF-1 were identified by using MCF-7 cells to screen for agents interfering with both ER-dependent gene expression and cell growth. Specifically, agonist- or antagonist-activated PPARγ was found to effectively block estrogen-mediated cell proliferation by suppressing SDF-1 gene expression (8). However, in contrast to progestins, PPARγ ligands blocked estrogen induction of SDF-1 in a specific manner, as there was no demonstrable effect on the expression of several other ER-regulated genes tested. In probing the mechanism, it was determined that PPARγ can interact directly with a negative regulatory sequence embedded within the Estrogen Response Element in the SDF-1 promoter, actively suppressing its activity (8). These studies highlight an important direct cross-talk between the ER and PPARγ signaling pathways and may provide the rationale for near term clinical evaluation of PPARγ ligands as chemotherapeutics and chemopreventatives for ER+/ PPARγ + cancers. In addition, however, we believe that PPARγ may represent a therapeutic target in ER-negative cancers; for example, SDF-1 was recently shown to be involved in the homing of ER- breast cancer cells to lung, suggesting agents targeting SDF-1 signaling may be anti-metastatic (9).

It was surprising to find that in BG-1 ovarian cells, Class I, yet not Class II agents were effective inhibitors of ER-mediated induction of SDF-1. However, while PPARγ is expressed in most tissues and cancer types (including ovary), we determined that BG-1 cells actually lack detectable PPARγ, providing an explanation for the observed results. We have therefore initiated screening for ovarian cancer cell lines expressing both ER and PPARγ, which would enable us to test the efficacy of PPARγ ligands in inhibiting ovarian cancer cell growth. Given that ovarian cancer cells and tumors are known to overexpress SDF-1 and receptor CXCR4, PPARγ may represent a therapeutic target in both ER+ and ER- ovarian cancers. Thus, we suggest that immunological evaluations of ovarian and breast tumors for ER and PR status should include analyses of PPARγ expression.

In summary, these studies provide evidence that progestins and PPARγ ligands may have utility in the treatment and/or prevention of ovarian and breast cancers by suppressing autocrine production of SDF-1. Thus, we suggest that targets within the ER/SDF-1/CXCR4/ PPARγ regulatory systems will be amenable to future cancer drug discovery.

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Key research accomplishments

1) We have accrued over 1,500 subjects to a prospective, population-based, case-control study of ovarian cancer in North Carolina. Blood and tissue samples and epidemiologic data have been accrued as well. Analyses of genetic susceptibility polymorphisms and molecular epidemiologic signatures are ongoing.

2) The +331G/A polymorphism in the progesterone receptor is protective against endometrioid/clear cell ovarian cancers.

3) We have shown that progestins markedly activate TGF-β signaling pathways in the ovarian epithelium in primates, and that these effects are highly associated with apoptosis. We are now performing studies *in vitro* designed to characterize the complex biologic effects of progestins and vitamin D analogues on apoptotic and TGF-β signaling pathways in ovarian epithelial cells. These findings will provide guidance in conducting a chemopreventive trial in chickens with these agents.

Reportable outcomes

1) The +331G/A polymorphism appears to be protective against endometrioid and clear cell ovarian cancers.

2) Combinations of progestins and vitamin D may act in an additive fashion to decrease growth of ovarian cancer cells.

Papers

1) Rodriguez GC, Nagarsheth N, Rex C. Bentley, Walmer DK, Cline M, Whitaker RS, Eisner P, Berchuck A, Dodge R, Adams M, Hughes CL: Progestin induction of apoptosis in the macaque ovarian epithelium is associated with differential regulation of transforming growth factor-beta. J Natl Cancer Inst 2002;94:50-60.

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Conclusions

The studies initiated by our program will enable us to define more homogeneous subsets of ovarian cancer based on epidemiologic and molecular characteristics, to identify women who are at increased risk for this disease and to develop chemopreventive strategies designed to decrease ovarian cancer incidence and mortality. We anticipate that much of our data will grow to maturity in the coming few years with continued support from the DOD Ovarian Cancer Research Program.

Appendices

Progesterone Receptor Promoter *+331A* Polymorphism is Associated with a Reduced Risk of Endometrioid and Clear Cell Ovarian Cancers

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Departments of [1]Obstetrics and Gynecology/Division of Gynecologic Oncology, [2]Community and Family Medicine, and [3]Surgery, Duke University Medical Center, Durham, North Carolina; [4]Centre for Genetic Epidemiology, University of Melbourne, Melbourne, Victoria, Australia; and [5]Population and Clinical Sciences Division and [6]Cancer and Cell Biology Division, Queensland Institute of Medical Research, Brisbane, Queensland, Australia

Abstract

Objective: **The progestagenic milieu of pregnancy and oral contraceptive use is protective against epithelial ovarian cancer. A functional single nucleotide polymorphism in the promoter of the progesterone receptor (*+331A*) alters the relative abundance of the A and B isoforms and has been associated with an increased risk of endometrial and breast cancer. In this study, we sought to determine whether this polymorphism affects ovarian cancer risk.**

Methods: **The *+331G/A* polymorphism was genotyped in a population-based, case-control study from North Carolina that included 942 Caucasian subjects (438 cases, 504 controls) and in a confirmatory group from Australia (535 cases, 298 controls). Logistic regression analysis was used to calculate age-adjusted odds ratios (OR).**

Results: **There was a suggestion of a protective effect of the *+331A* allele (AA or GA) against ovarian cancer in the North Carolina study [OR, 0.72; 95% confidence interval (95% CI), 0.47-1.10]. Examination of genotype frequencies by histologic type revealed that this was due to a decreased risk of endometrioid and clear cell cancers (OR, 0.30; 95% CI, 0.09-0.97). Similarly, in the Australian study, there was a nonsignificant decrease in the risk of ovarian cancer among those with the *+331A* allele (OR, 0.83; 95% CI, 0.51-1.35) that was strongest in the endometrioid/clear cell group (OR, 0.60; 95% CI, 0.24-1.44). In the combined U.S.-Australian data that included 174 endometrioid/clear cell cases (166 invasive, 8 borderline), the *+331A* allele was significantly associated with protection against this subset of ovarian cancers (OR, 0.46; 95% CI, 0.23-0.92). Preliminary evidence of a protective effect of the *+331A* allele against endometriosis was also noted in control subjects (OR, 0.19; 95% CI, 0.03-1.38).**

Conclusions: **These findings suggest that the *+331G/A* progesterone receptor promoter polymorphism may modify the molecular epidemiologic pathway that encompasses both the development of endometriosis and its subsequent transformation into endometrioid/clear cell ovarian cancer.** (Cancer Epidemiol Biomarkers Prev 2004;13(12):1–7)

Introduction

Epidemiologic studies have shown that both pregnancy and use of oral contraceptives dramatically reduce ovarian cancer incidence (1). Reduction in numbers of lifetime ovulations due to pregnancy or oral contraceptive use may decrease risk by reducing gonadotropin levels, oxidative stress, DNA replication errors, and inclusion cyst formation in the ovarian epithelium. In addition, whereas estrogens and androgens have been shown to increase ovarian cancer risk, both pregnancy and oral contraceptive use are characterized by a protective progestagenic hormonal milieu (1, 2). We have previously reported that oral contraceptives with high progestin potency were associated with a greater ovarian cancer risk reduction than those with low progestin potency (3). In addition, we have shown that progestins may reduce ovarian cancer risk by stimulating the apoptosis of genetically damaged ovarian epithelial cells that otherwise might eventually evolve a fully transformed phenotype (4, 5). This may account for the observation that the protective effect of pregnancy and oral contraceptives is far greater than the extent to which lifetime ovulatory cycles are reduced (1).

In view of the protective effect of progestins against ovarian cancer, progesterone receptor variants with altered biological activity may affect ovarian cancer susceptibility. A German group reported that an insertion polymorphism in intron G of the progesterone receptor was associated with a 2.1-fold increased ovarian cancer risk (6, 7). It was subsequently shown that this intronic *Alu*I insertion is in linkage disequilibrium with

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polymorphisms in exons 4 and 5. However, several subsequent studies have failed to confirm an association between these polymorphisms and ovarian cancer risk (8-12). In addition, there is little published evidence that this complex of polymorphisms, termed PROGINS, alters progesterone receptor function.

More recently, sequencing of the progesterone receptor gene has revealed several additional polymorphisms, including one in the promoter region (*+331G/A*; ref. 13). The *+331A* allele creates a unique transcriptional start site that favors the production of progesterone receptor B (PR-B) isoform over progesterone receptor A (PR-A; ref. 13). The PR-A and PR-B isoforms are ligand-dependent members of the nuclear receptor family that are structurally identical, except for an additional 164 amino acids at the NH_2 terminus of PR-B, but their actions are distinct (14, 15). The full-length PR-B functions as a transcriptional activator, and in the tissues where it is expressed, it is a mediator of various responses, including the proliferative response to estrogen or the combination of estrogen and progesterone (16). PR-A is a transcriptionally inactive dominant-negative repressor of steroid hormone transcription activity that is thought to oppose estrogen-induced proliferation. An association has been reported between the *+331A* allele and increased susceptibility to endometrial (13) and breast cancers (17). It was postulated that up-regulation of PR-B in carriers of the *+331A* allele might enhance formation of these cancers due to an increased proliferative response.

We used a case-control study design to explore whether the *+331G/A* polymorphism in the progesterone receptor promoter affects susceptibility to various histologic types of ovarian cancer in North Carolina. A second, independent, case-control study from Australia was examined to confirm associations seen in the North Carolina study.

Materials and Methods

Subjects

North Carolina Ovarian Cancer Study. Primary ovarian cancer cases enrolled in the study were identified through the North Carolina Central Cancer Registry, a statewide, population-based tumor registry, using rapid case ascertainment. Eligibility criteria for ovarian cancer cases include diagnosis since January 1, 1999, ages 20 to 74 years at diagnosis, no prior history of ovarian cancer, and residence in a 48-county area of North Carolina. Physician permission was obtained before an eligible case was contacted. The diagnosis of epithelial ovarian cancer (borderline or invasive) was confirmed by the study pathologist. The response rate among eligible cases was 82%. Nonresponders were classified as patient refusal (6.7%), inability to locate the patient (4.0%), physician refusal (3.5%), death (2.6%), or debilitating illness (1.6%). Population-based controls were identified from the same 48-county region as the cases and were frequency matched to the ovarian cancer cases based on race (Black and non-Black) and age (5-year age categories) using list-assisted random digit dialing. Potential controls were screened for eligibility and were required to have at least one intact ovary and no prior diagnosis of ovarian cancer. Seventy-three percent of controls identified by random digit dialing who passed the eligibility screening agreed to be contacted and were sent additional study information. Among those sent additional study information, the response rate was 68%. The study protocol was approved by the Duke University Medical Center Institutional Review Board and the human subjects committees at the North Carolina Central Cancer Registry and each of the hospitals where cases were identified. Trained nurse interviewers obtained written informed consent from study subjects at the time of the interview, usually in the home of the study subject. A 90-minute questionnaire was given to obtain information on known and suspected ovarian cancer risk factors including family history of cancer in first- and second-degree relatives, menstrual characteristics, pregnancy and breast-feeding history, hormone use, and lifestyle characteristics such as smoking, alcohol consumption, physical activity, and occupational history. A life events calendar, including marriage and education, was used to improve recall. Additionally, anthropometric descriptors (height, weight, waist, and hip circumference) were measured and blood samples (30 mL) were collected. Germ line DNA was extracted using PureGene DNA isolation reagents according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN). Analysis of data from the North Carolina study was limited to Whites. Data from 81 African American controls and 67 cases were excluded because of the low frequency of the polymorphism. Data were collected from 16 non-Black, non-Caucasian cases and 10 controls but were excluded because of the significant racial diversity and small size of this group.

Australian Study. Details of cases and controls included in the Australian study have been described previously (18). Briefly, the case sample consisted of 553 women with primary epithelial ovarian cancer ascertained as incident case subjects as part of a large population-based, case-control study from major gynecologic-oncology treatment centers in New South Wales, Victoria, and Queensland from 1990 to 1993 (n = 363) and from the Royal Brisbane Hospital, Queensland from 1985 to 1996 (n = 190). Histopathologic information regarding tumor behavior (low malignant potential or invasive), histology, stage, and grade was available for all women; information on potential or known ovarian cancer risk factors was ascertained by detailed questionnaire for the subset of cases in the population-based study and included age, ethnicity, country of birth, parity, oral contraceptive use, tubal ligation, hysterectomy, and age at menarche. Limited information ascertained from hospital records was also available for the Royal Brisbane Hospital patients and included age, ethnicity, and country of birth. Because blood samples were not collected from controls who participated in the ovarian cancer case-control study, an additional group of women, selected based on date-of-birth distribution to best match cases, were included in the analyses. The control sample consisted of 300 adult female unrelated monozygotic twins (one per pair), ages 30 to 90 years, recruited through the volunteer Australian Twin Registry for the Semistructured Assessment for the Genetics of Alcoholism study. This study reported participation rates of ~70% for monozygotic female twins and recruited individuals nationally from major cities in the eastern states of Australia. Limited information

ascertained by detailed questionnaires as part of the Semi-structured Assessment for the Genetics of Alcoholism study was available for these women to assess confounding and included age, ethnicity, country of birth, parity, and age at menarche. More than 90% of case and control subject groups were of northern European descent, and all subjects were from major cities in the eastern Australian states. Approvals were obtained from the ethics committees of the University of Melbourne, New South Wales Cancer Council, Anti-Cancer Council of Victoria, and Queensland Institute of Medical Research in Australia. Written informed consent was obtained from each participant. DNA isolation methods have been detailed elsewhere (18). Fourteen Australian cases ages <30 years were excluded from this analysis because no controls were ages <30 years. Additionally, four cases and two controls were excluded because they did not have *+331G/A* polymorphism results. Thus, the Australian sample used for this analysis consisted of 535 cases and 298 controls.

Genotyping of *+331G/A* Polymorphism. Allelic discrimination was done using the MGB primer/probe Taqman assay on the ABI Prism 7700 system. Details of the methods are described in the following sections.

North Carolina Study. Each 20 μL PCR reaction contained 18 pmol of forward primer 5′-CACGAGTTTGATGCCAGAGAAA-3′, 18 pmol of reverse primer 5′-GCGACGGCAATTTAGTGACA-3′, 4 pmol of G-allele probe (VIC)-CGGCTCcTTTATC-(MGBNFQ)-3′, 4 pmol of A-allele probe (FAM)-CGGCTCtTTTATCTC-(MGBNFQ)-3′ (200 nmol/L), 10 μL of 2× Taqman Universal Master Mix without AmpErase UNG (Applied Biosystems, Foster City, CA), and 25 ng of extracted leukocyte DNA. Cycling conditions were 95°C for 10 minutes followed by 40 cycles of 92°C for 15 seconds and 60°C for 60 seconds. Allelic discrimination was done in 96-plate format in the ABI Prism 7700 and analyzed using the ABI Prism 7700 software. Some samples in the North Carolina ovarian cancer study were subjected to sequencing to confirm results obtained using the Taqman assay. To do this, a 50 μL PCR reaction was done using forward primer 5′-AACTCAGCGAGGGACTGAGA-3′ and reverse primer 5′-GAGGACTGGAGACGCAGAGT-3′, 0.5 ng/μL genomic DNA, 0.5 nmol/L forward primer, 0.5 nmol/L reverse primer, 0.2 mmol/L deoxynucleotide triphosphate, 1.5 mmol/L $MgCl_2$ (Applied Biosystems), 1× Applied Biosystems PCR buffer, and 0.025 units/μL AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR conditions consisted of an initial denaturing step at 95°C for 12 minutes, 32 cycles of 94°C for 60 seconds, 55.0°C for 60 seconds, and 72°C for 3 minutes, and an extension step at 72°C for 10 minutes. Samples were held at 4.0°C until they were purified using QIAquick 96 vacuum filter plates (Qiagen, Germantown, MD) and finally eluted in 150 μL of 10 mmol Tris-HCl (pH 8.5). A sequencing reaction was done using 1 μL of purified product and 4.4 pmol of unlabeled forward primer in a BigDye Terminator Cycle Sequencing Reaction as described by the supplier (Applied Biosystems). Samples were analyzed on the ABI 3100 system and sequences determined using GeneScan software (Applied Biosystems).

Australian Study. Genotyping was done with Taqman methodology using identical probes as the North Carolina study. For detection and sequence confirmation of positive controls, a 381-bp product was amplified using the forward primer 5′-GTACGGAGCCAGCAGAAGTC-3′ and reverse primer 5′-ATCCTGTCGTCAGGGGAACT-3′. Denaturing high-performance liquid chromatography (Helix System, Varian Chromatography Systems, Walnut Creek, CA) was used to identify heterozygous GA individuals at 62°C recommended by the MELT program (http://insertion.stanford.edu/melt.html). Genotypes were confirmed by sequencing. Heterozygous GA PCR product was subcloned using the pGEM-T system to obtain G and A clones to use as control standards for the SDS allelic discrimination assay. The 15 μL PCR reaction contained 900 nmol/L of forward primer 5′-GCGACGGCAATTTAGTGACA-3′, 900 nmol/L of reverse primer 5′-TGCACGAGTTTGATGCCAGA-3′ (giving a 68-bp product), 150 nmol/L of A-allele probe, 200 nmol/L of G-allele probe, 1× Platinum Quantitative PCR SuperMix UDG (including passive reference ROX dye, Invitrogen, Melbourne, Victoria, Australia), and 15 ng of genomic or control sample that had been dried in 96-well plates. PCR was done using the ABI 7700 SDS PCR machine for 2 minutes at 50°C and 2 minutes at 95°C followed by 45 two-step cycles of 15 seconds at 92°C and 1 minute at 60°C.

Statistical Analysis. The genotype data were tested for Hardy-Weinberg equilibrium using the χ^2 goodness-of-fit test. Multivariate unconditional logistic regression models, adjusted for age, were used to estimate odds ratio (OR) and 95% confidence interval (95% CI) for the association between polymorphism and epithelial ovarian cancer for all cases as well as for various disease categories. Potential confounders including menopausal status, tubal ligation, oral contraceptive use, body mass index, family history of breast or ovarian cancer in first- and second-degree relatives, and parity were individually adjusted for in the North Carolina data to determine if they changed the crude OR by ≥10%. Analysis stratified by each of these factors was also conducted to assess potential effect modification. We found no evidence of confounding by these factors and therefore felt it appropriate to combine the Australian and North Carolina data despite limited epidemiologic data in the Australian sample. The Breslow-Day χ^2 test was used to assess homogeneity of the results from the two study populations. Analyses involving the combined data set were based on a reanalysis of the raw data and were adjusted for study as well as age. All calculations were done using SAS 8.0 (SAS Institute, Inc., Cary, NC).

Results

The demographic features, epidemiologic risk factors, and pathologic characteristics of cases and controls in the North Carolina (Caucasians only) and Australian studies are shown in Table 1. Of note, the median ages of the cases and controls in both North Carolina and Australian studies are similar. Caucasian women with ovarian cancer in North Carolina were more likely to have used oral contraceptives compared with Australian women with ovarian cancer (67% and 49%, respectively). Invasive ovarian cancer cases comprised 77% of the North Carolina cases compared with 84% of the Australian cases. The *+331G/A* single nucleotide polymorphism in the promoter of the progesterone receptor initially was genotyped in samples from the North

Table 1. Demographics and pathologic characteristics of cases and controls

	North Carolina Study		Australian Study	
	Cases (n = 438), n (%)	Controls (n = 504), n (%)	Cases (n = 535), n (%)	Controls (n = 298), n (%)
Age*				
Median (range)	55 (20-74)	53 (20-75)	59 (30-95)	50 (30-94)
Menopause status				
Premenopausal/perimenopausal	166 (38)	204 (40)		
Postmenopausal	272 (62)	300 (60)		
Parity*				
0	93 (21)	68 (13)	71 (20)	38 (13)
1	73 (17)	72 (14)	51 (15)	20 (7)
2	146 (33)	210 (42)	103 (30)	75 (25)
≥3	126 (29)	154 (31)	123 (35)	160 (55)
Oral contraceptive use*				
Yes	294 (67)	349 (69)	169 (49)	
No	144 (33)	155 (31)	179 (51)	
Tumor behavior				
Borderline	102 (23)		87 (16)	
Invasive	336 (77)		448 (84)	
Tumor stage†				
1	160 (37)		166 (31)	
2	33 (8)		42 (8)	
3	224 (52)		276 (52)	
4	14 (3)		43 (8)	
Tumor histology				
Serous	270 (62)		318 (59)	
Endometrioid	56 (13)		63 (12)	
Mucinous	49 (11)		61 (11)	
Mixed cell	1 (0)		36 (7)	
Clear cell	23 (5)		32 (6)	
Other	39 (9)		25 (5)	

NOTE: Fourteen Australian cases ages <30 years were excluded from the entire analysis because no controls were ages <30 years.
*Parity use not known for 187 Australian cases and 298 Australian controls. Oral contraceptive use not known for 187 Australian cases and 5 Australian controls.
†Stage not known for eight Australian and seven NC cases.

Carolina Ovarian Cancer study using a Taqman assay. In 91 samples in which there was some ambiguity regarding the genotype using the Taqman assay, DNA sequencing was done for confirmation, and in all cases, the original genotypes were confirmed. The *+331A* allele was found in 59 of 504 (11.7%) Caucasian controls and the distribution of genotypes was in Hardy-Weinberg equilibrium (P = 0.53). Among individuals who reported their race to be African American, only 1 of 81 (1.2%) controls and 0 of 67 with ovarian cancer carried the *+331A* allele. In view of the rarity of the *+331A* allele in African Americans, these subjects were excluded from analyses of the association with ovarian cancer risk.

There were very few *+331A* homozygotes and these were combined with GA heterozygotes in calculating crude and age-adjusted ORs (Table 2). In the North

Table 2. Association between *+331G/A* polymorphism and risk of invasive and borderline epithelial ovarian tumors

Genotype	Borderline and invasive cases, n (%)	Controls, n (%)	OR* 95% CI	Invasive cases, n (%)	Controls, n (%)	OR* 95% CI
North Carolina study						
	n = 438	n = 504		n = 336	n = 504	
GG	400 (91.3)	445 (88.3)	1.00 (reference)	307 (91.4)	445 (88.3)	1.00 (reference)
AG	37 (8.4)	58 (11.5)		28 (8.3)	58 (11.5)	
AA	1 (0.2)	1 (0.2)		1 (0.3)	1 (0.2)	
AG/AA	38 (8.7)	59 (11.7)	0.72 (0.47-1.10)	29 (8.6)	59 (11.7)	0.72 (0.45-1.15)
Australian study						
	n = 535	n = 298		n = 448	n = 298	
GG	483 (90.3)	266 (89.3)	1.00 (reference)	407 (90.8)	266 (89.3)	1.00 (reference)
AG	48 (9.0)	30 (10.1)		37 (8.3)	30 (10.1)	
AA	4 (0.7)	2 (0.7)		4 (0.9)	2 (0.7)	
AG/AA	52 (9.7)	32 (10.7)	0.83 (0.51-1.35)	41 (9.2)	32 (10.7)	0.76 (0.46-1.27)

NOTE: Fourteen Australian cases ages <30 years were excluded from the entire analysis because no controls were ages <30 years.
*ORs adjusted for age. For combined data, ORs are adjusted for the study as well.

Carolina sample, there was a suggestion that the *+331A* allele was associated with a modest reduction in risk of both borderline tumors and invasive ovarian cancers (OR, 0.72; 95% CI, 0.47-1.10). Samples from the Australian study were genotyped independently and 10.7% of controls were found to carry the *+331A* allele. The distribution of genotypes in controls was found to be in Hardy-Weinberg equilibrium (P = 0.27). Although not statistically significant, a similar inverse association with invasive ovarian cancer risk was observed (OR, 0.83; 95% CI, 0.51-1.35; Table 2). Excluding the borderline ovarian cancers revealed little change in the point estimates of the association between the *+331A* allele and ovarian cancer for either North Carolina or Australian comparisons (Table 2).

Analyses by histologic subtype for the North Carolina and Australian studies are presented in Table 3. A modest, nonsignificant decreased risk was observed in the North Carolina study among carriers of the *+331A* allele for the common serous histologic type (OR, 0.80; 95% CI, 0.49-1.29), but there was a striking decreased risk of endometrioid cancers (OR, 0.43; 95% CI, 0.13-1.40). Because endometrioid and clear cell ovarian tumors are thought to have a common etiology due to their association with endometriosis (19), these cases were combined to examine the overall association with the *+331A* allele of the progesterone receptor promoter polymorphism (OR, 0.30; 95% CI, 0.09-0.97). No consistent effect was observed between the *+331A* allele and mucinous ovarian cancers. These relationships according to histologic subtype were not modified by age, parity, history of oral contraceptive use, body mass index, or family history of breast/ovarian cancer.

In the Australian data, the protective effect of the *+331A* allele was most pronounced in endometrioid cancers (OR, 0.51; 95% CI, 0.17-1.53). The OR (95% CI) for the combined endometrioid and clear cell group was 0.60 (0.25-1.44). The Breslow-Day χ^2 test was indicative of homogeneity between the North Carolina and the Australian studies with respect to the association between the *+331A* allele and risk of ovarian cancer overall (P = 0.58) as well as endometrioid and clear cell ovarian cancer (P = 0.24). Pooling data from both North Carolina and Australian studies and controlling for study site, the age-adjusted OR (95% CI) for the association between the *+331A* allele and endometrioid/clear cell cancers combined (n = 174; 166 invasive, 8 borderline) was 0.46 (0.23-0.92).

Associations between the *+331A* allele and endometriosis were examined in the North Carolina study because endometriosis is known to increase the risk of endometrioid and clear cell ovarian cancers (19). The rate of self-reported endometriosis was 12.6% in cases and 7.5% in controls, similar to other reports in the literature (19). Endometriosis was associated with an increased risk of ovarian cancer (OR, 1.76; 95% CI, 1.14-2.72). This was mostly attributable to an increased risk of endometrioid/clear cell cases (OR, 3.87; 95% CI, 2.09-7.17; non–endometrioid/clear cell cases OR, 1.36; 95% CI, 0.84-2.20). Preliminary evidence of a protective effect of the *+331A* allele of the progesterone receptor polymorphism against endometriosis was also noted in control subjects (OR, 0.19; 95% CI, 0.03-1.38).

Table 3. Association between progesterone receptor polymorphism and risk of invasive and borderline epithelial ovarian tumors by histologic type and study

	GG	AG	AA	AG/AA (%)	OR* (95% CI)
North Carolina study					
Controls	445	58	1	59 (11.7)	1.00 (reference)
Serous	244	26	0	26 (9.6)	0.81 (0.50-1.32)
Mucinous	44	5	0	5 (10.2)	0.80 (0.30-2.14)
Endometrioid	53	3	0	3 (5.4)	0.43 (0.13-1.40)
Clear cell	23	0	0	0 (0.0)†	
Endometrioid/clear cell	76	3	0	3 (3.8)	0.30 (0.09-0.97)
Mixed	1	0	0	0 (0.0)†	
Other	35	3	1	4 (10.3)	0.86 (0.29-2.51)
Australian study					
Controls	266	30	2	32 (10.7)	1.00 (reference)
Serous	285	31	2	33 (10.4)	0.89 (0.52-1.52)
Mucinous	55	6	0	6 (9.8)	0.91 (0.36-2.27)
Endometrioid	59	3	1	4 (6.3)	0.51 (0.17-1.53)
Clear cell	29	3	0	3 (9.4)	0.83 (0.24-2.92)
Endometrioid/clear cell	88	6	1	7 (7.4)	0.60 (0.25-1.44)
Mixed	32	3	1	4 (11.1)	1.01 (0.32-3.17)
Other	23	2	0	2 (8.0)	0.73 (0.15-3.44)

*ORs are according to genotype (AG/GG) compared with the reference group genotype (GG) and are adjusted for age and corresponding study.
†Sample size too small to calculate.

Discussion

Epidemiologic studies have long suggested that heredity plays a role in ovarian cancer predisposition (20). Two high-penetrance ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, have been identified, defects that increase ovarian cancer risk dramatically (21, 22). It is estimated that up to ~10% of ovarian cancers are attributable to inherited mutations in *BRCA1* and *BRCA2* (22), but <0.5% of individuals in most populations carry these mutations. Although other high-penetrance genes may exist, low-penetrance polymorphisms are likely to contribute to the burden of ovarian cancers classified as sporadic. The PROGINS polymorphism in the progesterone receptor was initially reported to increase ovarian cancer risk (6, 7), but this finding was not confirmed by subsequent studies, including the North Carolina Ovarian Cancer study (8-12). The potential for false-positive results in association studies is now widely accepted, and confirmation in independent populations is now deemed critical prior to concluding that a true association exists (23).

A functional polymorphism in the progesterone receptor promoter (*+331A*) that favors production of PR-B is carried by ~11% of the Caucasian population (13). The group that described this polymorphism has reported associations between the *+331A* allele and increased risks of endometrial cancer (OR, 1.9; 95% CI, 1.10-3.29; ref. 13) and breast cancer (OR, 1.33; 95% CI, 1.01-1.74; ref. 17). The most striking increased risks were observed in obese women (endometrial cancer OR, 4.71; breast cancer OR, 2.30), suggesting an interaction between the polymorphism and the endogenous hormonal milieu. Because there were few rare allele homozygotes, these associations were based on a model in which heterozygotes were pooled with rare allele

homozygotes. It was postulated that the rare allele of this polymorphism may increase endometrial and breast cancer risks by enhancing PR-B-mediated proliferation in response to estrogen.

In the population-based North Carolina Ovarian Cancer Study, risk analyses were confined to Caucasian subjects because of the rarity of *+331A* allele in African American women. Among Caucasian women, we observed a weak protective effect of the *+331A* allele against ovarian cancer (borderline and invasive). Histologic subtype analysis revealed that there was a weak, nonsignificant decrease in risk of serous cancers, which are the most common subtype, whereas a stronger decreased risk for endometrioid cancers was observed. This association became even stronger and statistically significant after combining endometrioid and clear cell cancers, with about a two-thirds reduction in risk (OR, 0.30; 95% CI, 0.09-0.97) in carriers of the *+331A* allele, although the 95% CIs are wide suggesting the instability of the estimate. In view of the potential for false-positive results in association studies of genetic polymorphisms, we sought to confirm our findings in the Australian study. The frequency of the *+331A* allele among Caucasian controls varied by >1% between Australian and North Carolina studies and controls reported in the Nurses' Health Study (13, 17). The Australian study was not a population-based, case-control study and fewer data were available regarding risk factors. Nevertheless, the results of the Australian study were similar to those of the North Carolina study, with a modest overall protective effect that was most pronounced for endometrioid cancers (OR, 0.51; 95% CI, 0.17-1.53). Age was not associated with genotype and adjusting for age had minimal effect on the ORs reported in this article.

Serous and endometrioid/clear cell ovarian cancers share many of the same risk factors, such as parity and oral contraceptive use, but there is evidence to suggest that differences exist in their etiology, molecular pathogenesis, and clinical behavior. For example, there are differences between these histologic subtypes with respect to behavior (borderline versus invasive) and stage that likely reflect etiologic heterogeneity. In addition, mutations in *BRCA1* and *BRCA2* predispose primarily to serous cancers (24), which arise from epithelial cells that line the ovarian surface or underlying inclusion cysts. In contrast, it is thought that some, if not all, endometrioid and clear cell cancers arise from deposits of ovarian endometriosis (19). Coexistent endometriosis is commonly noted in women with ovarian endometrioid/clear cell cancers, and a strong association between endometriosis and these cancers has been reported in epidemiologic studies. Because endometriosis is likely to be underdiagnosed, the relationship between endometriosis and clear cell/endometrioid ovarian cancers may be stronger than noted in case-control studies.

The finding that the *+331A* allele was associated with a decreased risk of endometrioid and clear cell ovarian cancers was somewhat unexpected in view of prior reports of an increased risk of endometrial and breast cancers in carriers of the *+331A* allele (13, 17). However, these three diseases differ with respect to associated risk factors and predisposing hormonal milieu. Endometriosis is associated with endometrioid and clear cell ovarian cancers (19) but does not increase endometrial or breast cancer risk. In contrast, oral contraceptives are protective against all histologic types of epithelial ovarian cancer as well as endometrial cancers (1) but may increase breast cancer risk (25). In view of these significant differences in etiology, it is not surprising that predisposition to these cancers is affected differentially by the progesterone receptor promoter polymorphism.

PR-A and PR-B are both expressed in the ovarian (26), endometrial (27), and breast epithelium (28), and the relative expression of the isoforms is frequently altered during malignant transformation. In the present study, the *+331A* allele of the progesterone receptor promoter polymorphism was protective against endometrioid and clear cell ovarian cancers. We also observed preliminary evidence that this polymorphism may protect against endometriosis, the precursor of many of these cancers. Endometriotic implants have been shown to express only the PR-A isoform (27), and it has been suggested that the absence of PR-B may account for the lack of appropriate cycling of these glands. In normal cycling endometrium, PR-A expression is predominant during the proliferative phase, whereas a shift toward PR-B occurs with differentiation in the early secretory phase (29). Because the *+331A* allele of the progesterone receptor promoter polymorphism favors production of the PR-B isoform, it is possible that this might prevent the PR-A/PR-B imbalance in endometriotic implants and protect against the growth and spread of endometriosis to the extent that it becomes clinically apparent. The reduced risk of endometrioid and clear cell cancers in women with the *+331A* allele might be attributable to a lower likelihood of carriers developing more extensive endometriosis, which serves as a precursor for these cancers. In contrast to the pathogenic model proposed for endometriosis in which the *+331A* allele counters an abnormal imbalance in the PR-A/PR-B ratio in normal breast and endometrial tissues, the polymorphism may create an imbalance that enhances both the proliferative response to estrogen and cancer risk.

The literature is fraught with false-positive association studies of genetic susceptibility polymorphisms, but several features mitigate the likelihood of this in the present study. First, the known protective benefit of progestins against ovarian cancer provides a preexisting biological plausibility for the observed association. In addition, the finding that the *+331A* allele is protective against both endometrioid/clear cell cancers and their precursor lesion (endometriosis) is also supportive. Confirmation of the positive association obtained in the North Carolina study by the Australian study also represents an additional critical validation step. Finally, unlike many polymorphisms that lack known functional significance, the *+331A* allele is known to increase transcription of PR-B *in vitro* (13).

Despite the agreement between North Carolina and Australian data, the 95% CIs of the latter study are relatively wide. Furthermore, the control subjects in the Australian study were not collected in the context of an ovarian cancer study. However, allele frequencies in the Australian controls were similar to those seen in Caucasian controls in the North Carolina study. Another limitation of this study is that the number of cases of the less common histologic types was relatively modest,

limiting the power to draw definitive conclusions. Additional studies are needed to confirm the protective effect of the *+331A* allele against endometrioid and clear cell ovarian cancers.

In summary, the *+331A* allele of the progesterone receptor promoter polymorphism is carried by about one in nine Caucasian women and is associated with a decrease in risk of endometrioid and clear cell ovarian cancers. We also obtained preliminary evidence in support of a protective effect against endometriosis. These findings suggest that the *+331G/A* progesterone receptor promoter polymorphism may modify the molecular epidemiologic pathway that encompasses both the growth of endometriosis and its subsequent transformation into endometrioid/clear cell cancers. This study provides evidence for the existence of low-penetrance ovarian cancer susceptibility polymorphisms. If multiple polymorphisms are identified that either increase or decrease the risk of various histologic types of ovarian cancer, this might be used in the future for risk stratification that would facilitate screening and prevention strategies.

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